GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

VOLUME XIX
PAPERS 480 - 486

1955

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Official Organ of the Society for the Study of Development and Growth

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GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

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VOLUME XIX, NUMBER 1

PAPERS 463-466

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MARCH, 1955 **$6.00 A YEAR**

Entered as second class matter on October 20, 1941, at the Post Office,

GROWTH

A journal for the coordination of studies of increase and development as general properties of nature. Published by contributors and subscribers on a cooperative, non-profit, non-salary basis. The field of investigation is primary and work will be published in which the approach is through mathematics, physics, chemistry, genetics, embryology, philosophy, and anthropology; just as will studies of development in its several expressions, and of mass increase and the part played therein by nutrition, vitamins, and hormones.

It is published four times a year during March, June, September and December at $6.00 per volume (outside U. S. and Canada, $6.50). Single numbers $2.00. There will be one volume each year.

All manuscripts and subscriptions should be sent to: The Editors of Growth, Lankenau Hospital Research Institute, Fox Chase, Philadelphia 11, Pa.

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Growth, 1955, **19**, 1-8.

DIFFERENCES IN RESPONSE TO STYRYL QUINOLINE COMPOUNDS EXHIBITED BY NATIVE NEOPLASMS IN INBRED ANIMALS[1,2]

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(Received for publication February 7, 1955)

In studies concerning the anti-tumor activity of over 1000 compounds (1, 2, 3, 4, 5, 6), a number were found which showed considerable inhibitory activity when tested against rat and mouse sarcomas. Two hundred and thirty-five diaryl- and triarylmethane dyes (7) and a number of styryl pyridine and styryl quinoline compounds (8) were tested against Ehrlich mouse ascites tumor. When the results were evaluated by the criteria of *longevity* (the number of treated mice dying with tumor 20-60 days after the untreated controls had died from tumor), *resistance* (number of treated mice dying without tumor 2-10 days after the untreated controls had died from tumor) and *survivors* (number of treated mice that survived for 20-60 days or more and still remained free from tumor), it was seen that many of the compounds possessed some tumor-inhibitory activity. None, however, proved to be 100 per cent active in prolonging the life of the treated mouse or in promoting resistance to the growth of the ascites tumor.

In studies of the anti-tumor action of 9 styryl pyridine and 11 styryl quinoline compounds tested against neoplasms growing in rats and

[1] This investigation was supported in part by research grants C-1592(C) and C-285(C6) from the National Cancer Institute, Department of Health, Education and Welfare and in part by a grant from the Medical Research Foundation.
[2] The synthesis of the compounds used in this study was supported in part by the Frederick Gardner Cotrell grant to Carson-Newman College from the Research Corporation and in part by funds from the Medical Research Foundation.

mice, a number of styryl quinolines were found which exhibited powerful tumor-inhibitory properties against rat lymphoma No. 8 (Table 1). Six of them caused regression of the lymphoma in a high percentage of the treated rats.[3] Two of them, 4-(p-dimethylaminostyryl) quinoline methiodide and methochloride, described by Hughes, Bates, Bahner and Lewis (9), were found effective not only in preventing growth, but also in causing regression of lymphoma No. 8 in 100 per cent of the treated rats without apparent injury to the host animal. Large lymphomas (50 x 30 x 20 mm.) disappeared when the host was given food containing 0.3 per cent of either of these compounds.

The size of the tumor and the number of days it had grown prior to treatment had little influence on the final results. Even rats in terminal stage recovered their vitality and the lymphomas disappeared, provided the animals were able to ingest the treated food. When mice and rats bearing other types of tumor were used to test the anti-tumor properties of the styryl quinoline compounds, the results proved less satisfactory.

The relationship of the host animal to the neoplasm exerts a significant influence on the results achieved (10). Observations concerning the anti-tumor action of benzo(a)phenoxazines and of triethylene melamine (2, 5) show that tumor-bearing rats and mice may fail to respond in the same way to a given agent. Rats of one strain may respond to tumor-inhibitory treatment quite differently from rats of another strain (11). It, therefore, seemed of moment to compare the response to styryl quinoline compounds of the closely related rat neoplasms which originated in rats of the Wistar inbred strain, and have proved to be 100 per cent transplantable in these rats.[4]

It is conceivable that compounds administered orally or parenterally may become changed in the host animal or in the growing tumor itself. For this reason, the response of other native rat tumors was compared with that of lymphoma No. 8, not only when the two neoplasms were growing in separate rats of the same age, sex and strain, but also when growing in separate sites or the same site in the same animal, and when tiny fragments of sarcoma tissue were implanted

[3] See starred compounds in Table 1.
[4] Frequently referred to as Lewis strain of inbred rats.

into the central region of large lymphomas immediately prior to treatment of the host animal with the styryl quinoline compounds.

Material and Methods

Lymphoma No. 8 arose in a submaxillary lymph node of a female rat of the Wistar inbred strain, and proved to be 100 per cent transplantable in rats of this strain (12). The neoplastic cells metastasize to the lymph glands of the host and appear in the blood stream as early as 18 hours after subcutaneous or intraperitoneal implantation of lymphoma No. 8 tissue in the host animal. The neoplastic disease can be transmitted to normal rats by implants of spleen, lymph nodes and blood from the diseased rat as well as by direct subcutaneous implantation of lymphoma tissue. Sarcomas growing in rats bearing lymphoma No. 8 became infiltrated with lymphomatous cells from the blood stream. The growth pattern of the two neoplasms was not antagonistic; transplantation of the infiltrated sarcoma resulted in mixed tumors which remained partly lymphoma and partly sarcoma through five successive transplanted generations in normal rats (13).

Five 100 per cent transplantable neoplasms which originated in rats of the Wistar inbred strain were available for these studies, namely, sarcomas No. 4, No. 10 and No. 13, adenocarcinoma No. 11 and lymphoma No. 8. Tumors No. 4 and No. 10 were induced by means of methylcholanthrene; the others arose spontaneously.

Amounts equivalent to the desired concentration of the compounds to be tested, were ground together with pulverized Purina chow in a ball mill for several hours. The concentration selected depended largely upon its effectiveness, toxicity and whether the test animal consumed normal amounts of the treated food (10 to 12 grams per rat and 3 to 5 grams per mouse per day). Test animals were housed separately and furnished with food and water ad libitum.

Of the compounds tested (Table 1), the three found most satisfactory for use in the present study were 4-(p-dimethylaminostyryl) quinoline and its methiodide and methochloride salts.[5] The concentrations selected for use in these experiments were 0.01 to 0.05 per

[5] Preliminary tests of the inhibitory activity of these styryl quinoline compounds (with the exception of 4M2M) were made by Bahner at the Oak Ridge Institute of Nuclear Studies.

cent of the 4-(p-dimethylaminostyryl) quinoline, 0.3 per cent of the methiodide and 0.23 per cent of the methochloride.

TABLE 1
Styryl Pyridine and Styryl Quinoline Compounds Tested Against Native Neoplasms in Inbred Mice and Rats

	2-styrylpyridine methiodide	
	2-(p-dimethylaminostyryl) pyridine	ethiodide
	" "	methiodide
	2-(p-diethylaminostyryl) pyridine	ethiodide
	" "	methiodide
	4-(p-diethylaminostyryl) pyridine	ethiodide
	" "	methiodide
	4-(p-dimethylaminostyryl) pyridine	ethiodide
	" "	methiodide
	4-methylquinoline methiodide	
*	2-(p-dimethylaminostyryl) quinoline	
*	4-(p-dimethylaminostyryl) quinoline	
	2-(p-diethylaminostyryl) quinoline	ethiodide
	" "	methiodide
	2-(p-dimethylaminostyryl) quinoline	ethiodide
	" "	methiodide
*	4-(p-diethylaminostyryl) quinoline	ethiodide
	" "	methiodide
*	4-(p-dimethylaminostyryl) quinoline	ethiodide
*	" "	methiodide
*	" "	methochloride

Results

Each of the three compounds, 4-(p-dimethylaminostyryl) quinoline (4M20), the methiodide (4M2M) and the methochloride (4M2MCl) proved to be 100 per cent effective against lymphoma No. 8. The 4M20 possessed more powerful anti-tumor properties than either 4M2M or 4M2MCl; lymphoma No. 8 disappeared within 3 to 6 days in the 26 rats treated with 0.01 to 0.05 per cent 4M20 and within 7 to 14 days in the 150 rats treated with 0.3 per cent 4M2M and the 90 rats which received 0.23 to 0.3 per cent 4M2MCl.

The rate of growth of the neoplasms in the individual control rats varied somewhat. Sarcomas No. 4 and No. 13 grew at about the same rate as lymphoma No. 8. The growth of sarcoma No. 10, however, was slower than that of lymphoma No. 8. Because of this, the sarcoma No. 10 were smaller (about 30 x 28 x 20 mm.) while the No. 4 and No. 13 sarcomas were about the same size as the lymphoma (38 x 26 x 22 mm.) in the 26 rats implanted with the two types of tumor for these experiments. The growth of the two neoplasms, one lymphoma No. 8 and the other a sarcoma, implanted into separate

sites or the same site in the same rat was not antagonistic; each grew in its characteristic manner.

The growth of adenocarcinoma No. 11 was extremely slow. It was necessary to implant it four to six weeks earlier than lymphoma No. 8 to secure tumors of comparable size for these studies.

When lymphoma No. 8 was implanted into a rat bearing adenocarcinoma No. 11, each exhibited its characteristic rate of growth, The adenocarcinomas increase slowly from a tumor of about 30 x 20 x 15 mm. in size to one about 40 x 30 x 20 mm. during the three weeks in which lymphoma No. 8 grew from the implanted pieces into a tumor about 50 x 40 x 20 mm. in size, and brought about the death of the host.

Adenocarcinoma No. 11 responded slowly to treatment with the styryl quinoline compounds. When 6 rats each bearing both lymphoma No. 8 and adenocarcinoma No. 11 were treated with 4M20, the lymphomas disappeared within 3 to 6 days while the adenocarcinomas required 18 to 30 days. Even longer treatment was needed to bring about regression of the adenocarcinomas in the 6 rats which received 4M2M or 4M2MCl, although the lymphomas growing in the same site disappeared within 7 to 14 days. In four instances, viable adenocarcinoma tissue persisted until the death of the host animal took place. The loss of weight suffered by the rats during the prolonged treatment necessary to bring about regression of moderately large adenocarcinomas rendered adenocarcinoma No. 11 unsuitable for this type of experimental study.

The results obtained in the experiments devised for a comparison of the response to anti-tumor compounds of other types of native neoplasms with that of lymphoma No. 8 indicated that the site of implantation did not alter the characteristic behavior of neoplasms in rats treated with 4M20, 4M2M and 4M2MCl. In 20 rats, each bearing both a sarcoma and a lymphoma, the lymphomas regressed completely while the sarcomas continued to grow progressively regardless of whether the two tumors were growing in the same or separate sites in the same rat. Even small fragments of sarcoma tissue implanted into large lymphomas immediately before treatment became vascularized and multiplied although surrounded by the atrophying circulation and degenerating cells of lymphoma No. 8.

Adenocarcinomas regressed slowly, persisting long after neighboring lymphoma cells had disappeared. Marked differences in the meta-

bolic pattern of the five neoplasms which originated in rats of this closely inbred strain became evident when the host animals were treated with these styryl quinoline compounds.

Necrosis did not occur in lymphoma No. 8, either in treated or untreated animals. With the exception of sarcoma No. 10, necrosis was seldom observed in sarcomas growing in the control animals. On the other hand, extensive necrosis was found in many of the sarcomas present in rats undergoing treatment. The dead material accumulated in the central portion of the tumor; many tumors which appeared to be growing were found to be composed largely of necrotic material with only a rim of viable tumor tissue. Biopsies taken from lymphomas in various stages of regression in the treated animals, however, presented a quite different picture. Small areas of degenerating cells with pycnotic nuclei were present on the outer border of the tumor; the central portion was composed of viable lymphomatous cells. Regressing lymphoma tissue appeared to be absorbed almost as soon as the cells became inactivated. Fenninger and Mider (14) discuss the difference in absorption of necrotic tumor material and regressing lymphosarcoma tissue.

Discussion

Greenstein (15) finds that the metabolic pattern of all tumors at any one stage tend to resemble each other more closely than normal tissues resemble each other or than tumors resemble normal tissue of the same histological pattern. Nevertheless, tumors of the same type located in different regions in the same host or in different hosts as well as different types of tumors differ in response to anti-tumor treatment (16, 17, 18). Burchenal (19) states that two acute leukemias, morphologically alike, giving similar histories and similar physical findings, may be biochemically different and react differently to a given agent.

Certain carcinomas and leukemias have been found to be sensitive to the carcinostatic influence of 8-azoguanine when growing in mice of one strain, and refractory in mice of another strain. Studies of the mechanism involved indicated that the azoguanine deaminase activity was high in the resistant neoplasms and low in the susceptible ones (20, 21). In studies of alcoholic extracts of neoplasms which originated in rats of the Wistar inbred strain, Seibert et al. (22) found a higher level of several constituents, fructose, ascorbic acid and or-

ganic phosphorus, ribose nucleic acid and desoxyribose nucleic acid) in extracts of lymphoma No. 8 than in extracts of the sarcomas. Klein and Klein (23) suggest that cells able to survive in a depleted medium may have less extracting nutritional requirements than those that fail to survive.

Why do these styryl quinoline compounds possess such powerful tumor destroying action against *large* lymphomas No. 8? Studies of tumor-bearing animals and human beings show that the level of various enzymes, vitamines, plasma proteins, amino acids and other substances differ in normal and tumor-bearing individuals (15, 24, 25, 26). The results of the present studies concerning the response of two neoplasms, one lymphoma No. 8 and the other a sarcoma, No. 4, No. 10 or No. 13, growing in the same or separate sites in the same animal indicate that even should the styryl quinolines become activated in the lymphoma-bearing animal or in the tumor tissue itself, the product formed does not apparently possess the same tumor destroying property toward sarcomas that it does toward lymphoma No. 8.

Summary

Studies of the response of the various neoplasms (three sarcomas, an adenocarcinoma and a lymphoma) which originated in rats of the Wistar inbred strain to 4-(p-dimethylaminostyryl) quinoline, its methiodide and methochloride salts, showed that each type of tumor responded in a different manner to treatment with these compounds. Lymphomas disappeared within 5 to 12 days in 100 per cent of the 300 treated rats; adenocarcinomas regressed within 18 to 30 days in the majority of the 16 treated rats; while sarcomas continued to grow progressively in all of the 26 rats treated with these compounds.

The results of experiments devised for a comparison of the response to styryl quinoline compounds of two tumors, one lymphoma No. 8 and the other an adenocarcinoma or a sarcoma, growing in the same or separate sites in the same rat, indicated that the site of implantation did not alter the characteristic behavior of the tumors. Lymphomas disappeared rapidly even when surrounded by growing sarcoma tissue; adenocarcinomas regressed slowly, persisting long after the lymphoma tissue had been absorbed. Sarcomas continued to grow, even fragments of sarcoma tissue implanted into large lymphomas became vascularized and multiplied although the circulation

of the surrounding lymphoma became atrophied and the lymphomatous cells disappeared.

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Growth, 1955, **19**, 9-18.

SENSITIVITY TO X-RAYS OF THE EARLY CLEAVAGE STAGES OF THE SNAIL *HELISOMA SUBCRENATUM*

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(Received for publication February 14, 1955)

Previous experiments (Bonham and Palumbo, 1951) showed that eggs of the freshwater snail *Radix japonica* (Jay) were more radiosensitive than the adults, suggesting the desirability of investigating the early cleavage stages.

Holmes (1900) determined cell lineage for *Planorbis*. Noland and Carriker (1946) studied the biology of *Lymnaea* under laboratory culture, and Raven (1945 and 1946) described its developmental pattern from oviposition to trochophore. Comandon and de Fonbrune (1935) compared prehatching development of several genera including *Lymnaea* and *Planorbis* through the use of time-lapse cinematography. Sparrow (1951) reviewed the general literature bearing on radiosensitivity of cells at mitosis and meiosis.

For the present study, eggs were irradiated chiefly in early cleavage stages and then photographed periodically to record progress of development to hatching. Stage of embryonic attainment was determined from the photographs and was related to dose of X-ray. Dr. Joseph P. E. Morrison of the U. S. National Museum identified the planorboid snail as *Helisoma subcrenatum* (Carpenter). Egg masses recently laid in the aquarium, usually on *Vallisneria* leaves, were removed with a thin blade to a glass dish or slide. By means of a hasty camera lucida sketch of the egg mass before X-raying, individual eggs were identified by position with reference to an oblique fold (upper left in Figure 1) presumed to terminate the egg string at the periphery of the mass. Usually more than half of the eggs in the mass were shielded from X-rays and served as controls. Certain eggs selected under the microscope were irradiated through a pinhole in a lead shield. X-rays were generated, for experiments prior to 1953,

Work performed under Contract Number AT(45-1)540 with the Atomic Energy Commission.

by a Picker deep therapy unit of the 2-valve Villard voltage doubling type with Machlett FCX tube operating at 200 pKV, 20 MA, with added filters of ¼ mm copper and 1 mm aluminum. This machine required a prolonged warm-up period. Later experiments were expe-

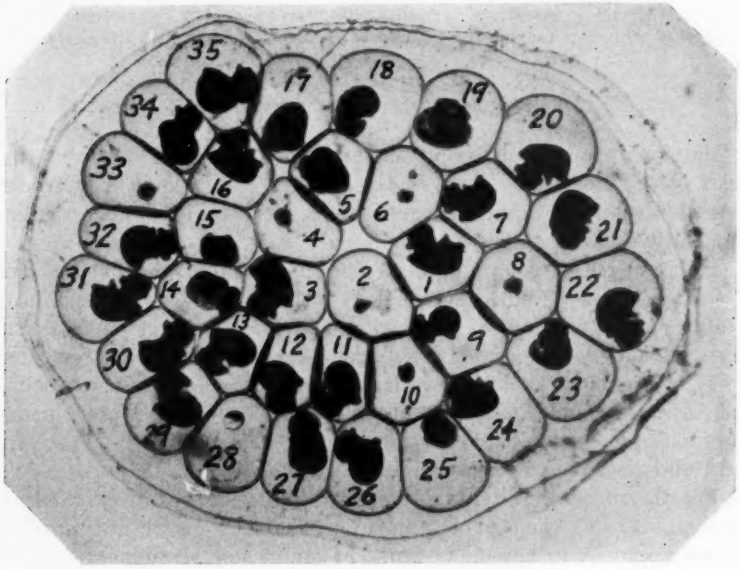


FIGURE 1

Photomicrograph taken with 35-mm camera on October 26, 1953 of a large 35-egg mass 7.4 mm long, laid on October 19 and selectively X-rayed at 50 pKV at a distance of 6 inches and at 400r per minute.

Stage irradiated	Egg number	Dose in r	Stage attained
Resting 2-cell	4	400	3
" "	2	800	2
" "	6	1600	1
" "	15	200	6
Metaphase, 2nd cleavage	14	160	6
" " "	10	160	4
" " "	25	200	9 small
" " "	8	400	3
" " "	28	400	3
" " "	18	200	8? or 9 small
" " "	33	400	3

The remaining 24 eggs were shielded serving as controls, and, with the possible exception of egg 19, all hatched.

dited by irradiating with a modified X-ray diffraction unit with a Machlett 0-2 beryllium window tube at 50 pKV fully rectified, 10 MA, and filtration only by the cellophane, plastic, or glass cover slip. The lead shield for the hard radiation was 3 mm thick, perforated by a 1.4-mm hole, and for the soft radiation, 0.2-0.3 mm thick with a 0.8-mm hole. Dosages were originally determined with a Victoreen medium energy 250r chamber. For soft X-rays corrections based upon readings with a low energy chamber were later applied for absorption by air, cover slip, fluid and albumen, and for distance. After these corrections, residual errors were subjectively estimated to be within 5% for hard, and 20% for soft, rays, the difference being due to the greater absorption of the soft radiation.

Photographs recorded the egg mass at the time of irradiation and at 1-3-day intervals. A small 35-mm camera supported by hand on the eyepiece of the microscope, either dissecting or compound, was preferred to photomicrographic equipment involving a stand. Fast film (Linagraph Ortho) helped stop blurring caused by motion of the embryos. From these pictures developmental stage was determined with errors not exceeding ½ day for stages up to 3 days or 1 day for later stages. Staging of very abnormal embryos was uncertain.

Time-lapse motion pictures revealed the details of early cleavage and growth.

TABLE 1

Timing of development to 5th cleavage, referred to earliest indentation of 1st cleavage.

Stage	Number of observations	Time in hours	Standard error
Oviposition	6	—2 to —3	—
1st polar body extrusion	9	—1.9	0.13
2nd polar body extrusion	13	—1.4	0.09
1st cleavage	29	0.0	0.00
2nd cleavage	19	1.6	0.08
3rd cleavage	15	3.4	0.13
4th cleavage	10	4.8	0.23
5th cleavage	4	5.8	0.69

Table 1 gives the timing at temperatures of 71°-74° F for events following oviposition as far as 5th cleavage. The earliest indentation of the egg was used as zero time because this stage was observed most frequently, and, as has been reported by Raven (1945:92) for *Limnaea stagnalis*, the time of oviposition is variable compared to events that follow. Freshly laid egg masses were sought, but only 2 of sev-

eral hundred observed were still accompanied by the depositing snail. Occasionally masses were found where none had been shortly before. Such observations suggest that oviposition usually precedes the first cleavage by 2-3 (2.2) hours.

Assignment of numbers designating the various stages through hatching facilitated quantifying of results. Incubation conveniently divides into 9 approximately equal stages terminating as:

1. blastula
2. gastrula
3. trochophore
4. early veliger
5. late veliger
6. early shelled embryo
7. mid-shelled embryo
8. late shelled embryo
9. hatched snail

Comparison of the photographic record with a set of reference pic-

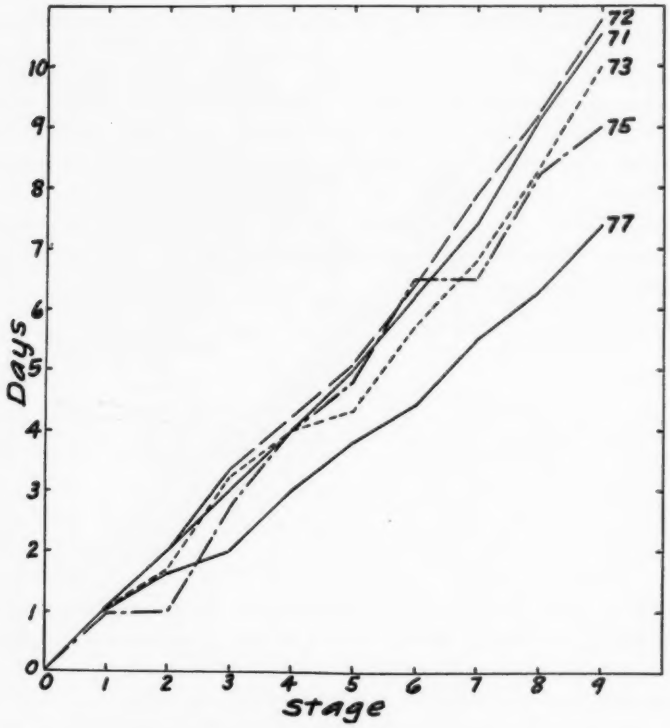


FIGURE 2
Prehatching developmental rate of eggs at various temperatures from 71° to 77°F.

tures, coupled with the writer's experience, determined the stage to which each embryo was subjectively assigned.

The influence of temperature over the range 71°-77° F upon developmental rate of 376 unirradiated control eggs appears in Figure 2, each embryo supplying 1-8 observations. Hatching occurs in 7.5 days at 77° F, but requires 10-11 days at 71°-73°. Regression of number of days required for hatching upon 6 bimonthly average temperatures in degrees Fahrenheit is:

$$Y = 50.41 - 0.555\,X$$

Correlation coefficient for these 6 points is 0.946. $P < 0.01$.

Because the mitotic phase is known to influence susceptibility of the cell to radiation damage, it was desirable to relate the invisible mitotic conditions to the microscopic appearance of the living egg, relying mainly upon shape. Eggs in the 1- to 4-cell stages were stained with aceto-orcein under sufficient cover slip pressure almost, or barely, to rupture the egg membrane. In these temporary mounts lasting only a few hours, the chromosomes were examined by oil-immersion phase microscopy using camera lucida and photography as aids in recording. Scarcity of published information on chromosome numbers of planorboid snails (Makino in 1951 lists no data) prompts the inclusion here of Figures 3 and 4 showing respectively polar and equatorial views of metaphase. Microscopic observation of these two preparations supported a count of 36 as the diploid chromosome number. The outline of these two eggs at the time of staining was eliptical preparatory to the first cleavage. The cleavage plane first appeared at the animal pole of other eggs in the same mass about 7 minutes later. Stained specimens at this time were in late anaphase.

Control eggs totaled more than half of the eggs in the masses. In the evaluation of radiation effects 8 experiments or 18% had to be excluded from consideration because of the failure of most of the control eggs in those masses to hatch. In the experiments analyzed almost all of the controls hatched, and those that failed to hatch attained stages from 1 to 9, mostly the later stages. The entire control development averaged stage 8.9.

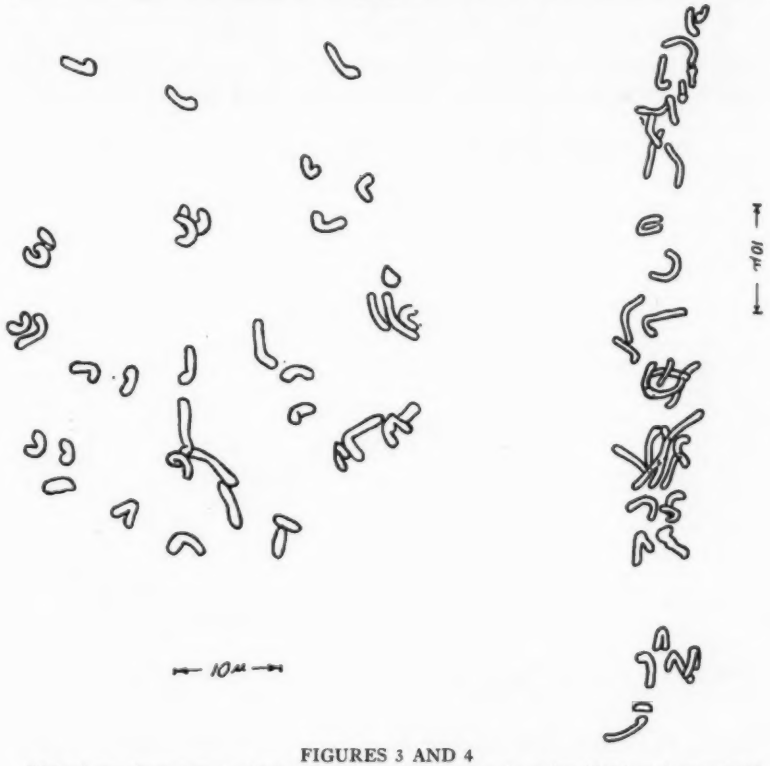
Table 2 shows for soft (50 pKV), and Table 3 for hard (200 pKV), X-rays the degree of development expressed numerically as stage attained, following various dosages administered in the 1- or 2-cell stage for the resting as compared to the mitotic condition. "Mitotic" in-

TABLE 2

Stage of prehatching development attained after irradiation with soft (50 pKV) X-rays to eggs in 1- and 2-cell stages during resting as contrasted with mitotic conditions.

Resting			Mitotic		
Dose in r	Stage attained	Frequency	Dose in r	Stage attained	Frequency
1680	2	1	1350	1	1
1580	9	2	900	2	1
1540	1	1	810	2	2
810	2	1	810	1	1
810	3	1	720	1	3
810	9	3	720	5	1
810	1	1	400	2	1
800	7	2	400	6	3
800	1	1	400	4	1
720	1	2	360	1	3
600	1	1	360	3	1
600	7	2	360	2	2
600	3	1	250	6	1
400	2	1	210	3	1
400	9	3	200	2	1
400	6	2	200	8	1
400	4	1	200	6	1
400	5	1	190	9	1
390	2	1	180	5	1
360	2	2	180	9	1
360	7	1	180	8	1
300	7	1	170	1	1
300	9	2	150	9	1
300	6	2	140	3	1
300	3	1	100	7	1
250	9	4	80	9	1
200	9	3	50	9	2
200	4	1			
190	9	8		total	36
180	4	1			
180	7	1			
170	9	2			
150	7	1			
150	9	2			
140	6	1			
80	9	1			
50	9	2			
30	9	1			
30	8	1			
	total	66			

cludes only those eggs irradiated less than 10 minutes either before or after the earliest indication of cleavage, and is thought to involve phases from late prophase to anaphase. Greater radiosensitivity is apparent in mitotic than in resting eggs. Table 2 indicates an LD_{50} to



FIGURES 3 AND 4

Metaphase chromosomes during first cleavage as seen in polar (left) and equatorial (right) views with phase oil-immersion. Drawn with camera lucida.

hatching of 300r-400r for eggs X-rayed in the resting phase, and about 100r for those irradiated during the ensuing mitosis. Figure 5 presents graphically most of the data of Table 2, showing by regression lines the relationship of stage attained to dose for the range from 140r to 900r. For the resting condition,

$$Y = 17.837 - 4.5007 \log_{10} X$$

while for mitotic,

$$Y = 18.995 - 5.9847 \log_{10} X.$$

Correlation coefficients of the two relationships are respectively,

$r = -0.379$, for 55 degrees of freedom, and
$r = -0.574$, for 30 degrees of freedom,

both significant beyond the 1% level of P. Conclusions regarding LD_{50}'s with hard rays (Table 3) agree with those for soft rays except

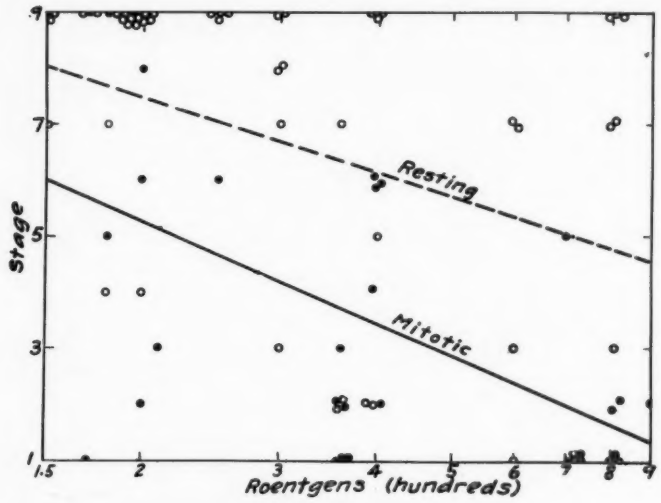


FIGURE 5

Relationship of stage attained to dose of soft X-rays administered during resting (open symbols) and mitotic (solid symbols) phases of first and second cleavage.

for the two snails that hatched from eggs given 380r during mitosis. Their small size at hatching may indicate latent radiation damage. No significant difference in effectiveness between hard and soft radiation is demonstrated.

Later embryonic stages evidence a lower radiosensitivity. Table 4 shows development attained by eggs receiving 100r-1100r during stages 3-6. Although hatching followed one dose of 1100r, the LD_{50} falls between 500r and 1000r.

It is probable that if the survival of the snails were to be followed

beyond hatching and into succeeding generations, the LD_{50} would be substantially lowered.

TABLE 3

Stage of prehatching development attained after irradiation with hard (200 pKV) X-rays to eggs in 1- and 2-cell stages during resting as contrasted with mitotic conditions.

Resting			Mitotic		
Dose in r	Stage attained	Frequency	Dose in r	Stage attained	Frequency
420	8	2	450	7	1
420	9	2	400	1	2
410	9	2	400	2	4
400	2	1	400	3	2
400	3	1	400	4	4
400	4	1	400	5	2
400	7	1	400	6	4
400	9	18	380	9 small	2
110	6	1	110	4	1
110	9	12	110	9	1
100	9	5	100	5	1
			100	9	4
	total	46		total	28

TABLE 4

Stage of prehatching development attained after irradiation with hard or soft X-rays during stages from trochophore to early shelled embryo (stages 3-6).

Dose in r	Stage attained	Frequency
100	9	3
200	9	1
500	9	1
1000	8	1
1000	6	2
1000	5	3
1100	9	1
2000	5	11

The marked difference in susceptibility between mitotic and resting cells, and between early cleavage mitotic and later stages (3-6) suggests that the particular mitotic phase irradiated is important in determining sensitivity of the organism. In irradiating the later stages it is unlikely that all cells will be undergoing the critical mitotic phases at the time of irradiation, and it may be assumed that those resting cells which survive the high dosages will help the organism carry on to hatching.

Summary

Prehatching mortality of snail eggs was used as a criterion of sensitivity to acute doses of X-radiation. Progress of egg development was recorded photographically. A timetable of early cleavages is presented. Temperature affects developmental rate so that hatching occurs in 11 days at 71° F, but in only 7.5 days at 77° F.

Stage of development was evaluated numerically permitting relationships to be established between dose and developmental attainment. In the 1- and 2-cell stages resting eggs withstood from 2 to 4 times as much radiation as cells undergoing mitosis. The LD_{50} to hatching was 300r-400r for resting cells and about 100r for mitotic.

Later embryonic stages were less sensitive. For the trochophore through early shelled embryo stages the LD_{50} was between 500r and 1000r.

Effectiveness of hard (200 pKV) X-rays did not differ significantly from that of soft (50 pKV).

Greatest sensitivity during first or second cleavage coincided with the earliest indentation of the cell. Aceto-orcein staining showed this to be at metaphase or anaphase. Stained metaphase preparations gave a diploid chromosome count of 36.

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STIMULATION OF TUMOROUS AGGREGATIONS IN DROSOPHILA BY ADDITION OF INDOLEACETIC ACID AND ADENINE TO CULTURE MEDIUM*

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(Received for publication February 21, 1955)

INTRODUCTION

The role of environment as a causative or modifying agent in the aggregation of melanotic tumorous masses in "tu" strains of *D. melanogaster* has been extensively studied (Hartnett '52, Hartung and Hartnett '51, Hartung '42, '47, '48, Herskowitz and Burdette '51, Mittler '52). Although these investigations have been largely successful in demonstrating that an hereditary propensity for the development of such masses may be played upon in a number of ways, and that normally occurring incidence may be varied by a number of factors, they have supplied little information regarding the nature of the "genetic propensity" or the mode of action of the environmental stimulation.

The present paper is a report on the effects of feeding 3 indoleacetic acid and adenine sulfate to tumorous and non-tumorous strains of *Drosophila*. The results differ from those of many previous investigations in that major effects in terms of tumor incidence are, to a degree at least, quantitative, and are noted in normally non-tumorous strains rather than in those characteristically showing some tumor incidence. While the induction of tumorous aggregations in wild strains in response to various mechanical, physical, and chemical stimuli has been more than occasionally reported (Harnley et al. '54, Hartung '42, Russell '40, Wilson '47, '48) it has proven to be a phenomenon less carefully studied than incidence in "tu" strains, and usually without the bounds of much approximating quantitative control.

Observations by the author (Hartung and Hartnett '51) that nor-

* A project supported in part by a grant from the Committee on Growth of the National Research Council acting in behalf of the American Cancer Society.

mally tumorous stocks of *D. melanogaster* showed highest incidence when grown on a medium containing cornmeal were confirmed by Hartnett ('52), Mittler ('52) and more recently by Harnley and coworkers ('54).

In an attempt to ascertain if reported "anti-niacin" factors in corn might be responsible for the stimulating effect of the medium, Hartnett studied the effects of adding massive doses of nicotinic acid and related compounds to the culture media of bw $mt^{A}vg$ tumor strains. Nicotinic acid compounds had little or no effect but tryptophane in large doses produced striking increases in incidence (Hartnett '52). These effects suggested that the stimulatory effect of cornmeal might be related to the presence of tryptophane or a tryptophane derivative. Indoleacetic acid, which is readily derived from tryptophane and known to be present in large quantities in sprouting corn appeared to be the compound of first choice in follow-up experiments. The fact that indoleacetic acid is also a very potent growth stimulating substance in plants added interest to the investigation of its possible role as a causative or modifying agent in the aggregation of tumorous masses.

As studies of the effects of indoleacetic acid added to culture media progressed, and it became evident that consistent stimulation of tumor aggregations was being effected in non-tumorous strains, the scope of the work was enlarged to include investigations into the effects of the addition of adenine to the medium also. Since adenine is in a sense an antagonistic substance to indoleacetic acid in plants, being a root stimulator and leaf inhibitor while indoleacetic acid is a leaf stimulator and root inhibitor, it became of interest to ascertain if a similar antagonistic effect might be noted in the matter of tumor stimulation or suppression.

Material, Methods, and Experimental Results

Experimental stocks of *D. melanogaster* included a tumor strain of moderately high incidence, bw $mt^{A}vg$ (Hartung '50) derived from bw tu stocks maintained at California Institute of Technology, a low incidence strain, st sr C^{v} ro Ca tu 36° also obtained from Cal. Tech., and two inbred "wild" strains, + Burlington (Vt.) and + Kingston (R.I.). A strain of *D. virilis* (*Maruyama*) obtained through the courtesy of Mr. T. Kaneshisa of Kobe U. was also utilized.

Up to the time of experimentation, stocks were routinely maintained

on standard cornmeal-molasses agar seeded with live Fleischman's yeast. Culture temperature was 25.° + .5°C. At the time of experimentation normal incidence in the bw strain was approximately 29-31% and in the st sr strain approximately 1%. Only very occasional tumors appeared in the + strains of *D. melanogaster* and none had been observed in the *D. virilis* stock.

Experimental culture medium was a dead yeast medium described by Thornton ('47). Ingredients are sucrose, agar, yeast, distilled water, ammonium sulfate, magnesium sulfate, tartaric acid, + potassium acid phosphate.

3-indoleacetic acid (Fisher Scientific) or adenine sulfate (Eastern Chemical) were added to dead yeast medium in measured quantities following autoclaving of the medium. Experimental larvae were grown in petri dishes prepared by heating stock bottles of sterile medium over boiling water until the medium became fluid, agitating the medium to insure mixing, and then pipetting 5 cc quantities under sterile precautions into sterile dishes. Between 80 and 150 larvae were usually reared in each dish.

Since tumorous masses aggregate during larval life it was considered desirable to have experimental animals hatch, and feed from time of hatching in the test culture dishes. This was accomplished by collecting eggs after the "bottle cap" method of Schweitzer ('35), scraping them off the agar cap into 95% ethyl alcohol, allowing them to remain immersed in alcohol for 30-40 minutes and then transferring them by sterile pipette from the alcohol into petri dishes of experimental medium. The period of immersion in alcohol effectively destroyed any yeast or other cells which might be adherent to the egg chorion. Thus the possibility of alteration of the test medium by the uncontrolled growth and metabolism of yeast or bacterial flora was eliminated. Egg collection jars and petri culture dishes were maintained at 25° ± .5°C throughout the experiments.

Larvae were permitted to develop to late third instar by which time tumor aggregation had generally progressed to its fullest, melanization was well along, and the tumor masses could be easily identified. They were then collected and examined for tumor masses under a dissecting microscope.

With a few exceptions a minimum of 500 larvae reared on each modification of the culture medium was examined. The exceptions were in cases where the larvae grew so poorly on the medium that

only very small numbers could be recovered from dishes. Percentage of tumors developing for each strain studied on each medium modification were calculated and compared.

Larvae of each of the four *D. melanogaster* strains, 2 tumorous and 2 wild strains as controls, were reared on the following media:

1) Dead yeast agar—0.1 gr. indoleacetic acid added to each 100 cc. medium.
2) Dead yeast agar—0.5 gr. indoleacetic acid added to each 100 cc. medium.
3) Dead yeast agar—0.1 gr. adenine sulfate added to each 100 cc. medium.
4) Dead yeast agar—0.5 gr. adenine sulfate added to each 100 cc. medium.

Since tumor strains and one control strain (+ Burl) did not grow well on medium to which 0.5 gr. adenine/100 cc. media had been added, a series of runs for all strains on 0.25 adenine/100 cc. medium was also set up.

As the recording and comparison of results made it clear that with only one exception relatively minor changes in tumor incidence were occurring in tumor strains, but that highly significant stimulation was occurring in non-tumorous controls on indoleacetic acid, additional cultures of wild stock were grown on 0.05 gr. indoleacetic acid/100 cc. medium and 0.2 gr. indoleacetic acid/100 cc. medium. These were added to ascertain if the stimulation was in any way proportional to increasing concentration of indoleacetic acid in the medium.

The results of this entire series of experiments are presented comparatively in tables 1 a, b, c, d.

It will be readily noted that except for the st sr strain at concentration of 0.5 gr. per 100 cc., indoleacetic acid has relatively little effect on incidence in the two tumor strains but is highly effective in stimulating tumor aggregation in normally non-tumorous strains. Further, this stimulating effect appears to be increased as the concentration in the medium increases. Maximum value of 51.9% incidence at 0.5 gr./100 cc. was achieved in the + Burlington strain, and of 28.1% at 0.5 gr./100 cc. in the + Kingston. In view of the fact that normal incidence in these strains is well under 1%, the results are quite striking.

The effects of adenine are less striking. Some suppression of inci-

dence appears in the case of the bw stock, and some tumor stimulation seems to be apparent in the wild stocks. Stimulation effects do not appear to increase with increasing concentration but are at maxi-

TABLE 1

Effects of varying concentrations of indoleacetic acid and adenine in medium on tumor incidence in "tu" and wild strains of *Drosophila melanogaster*.

Concentration	Total larvae scored	Number tumorous larvae	% tumor incidence
a) bw mt^A vg strain—normal incidence average 29-31%			
	3 indoleacetic acid		
.1gr/100cc	514	151	29.18
.5gr/100cc	596	157	26.34
	adenine sulfate		
.1gr/100cc	613	124	20.23
.25gr/100cc	273	58	21.24
.5gr/100cc	no growth	——	——
b) st sr C^v ro Ca tu 36° strain—normal incidence average 1-3%			
	3 indoleacetic acid		
.1gr/100cc	822	10	1.225
.5gr/100cc	1157	129	11.15
	adenine sulfate		
.1gr/100cc	717	3	0.418
.25gr/100cc	1049	21	2.09
.5gr/100cc	837	19	2.27
c) + Kingston strain—normal incidence average less than 1%			
	3 indoleacetic acid		
.05gr/100cc	511	6	1.17
.1gr/100cc	532	10	1.88
.2gr/100cc	554	100	18.05
.5gr/100cc	642	191	28.19
	adenine sulfate		
.1gr/100cc	981	20	2.036
.25gr/100cc	782	15	1.92
.5gr/100cc	515	5	0.97
d) + Burlington strain—normal incidence average less than 1%			
	3 indoleacetic acid		
.05gr/100cc	1010	7	0.69
.1gr/100cc	609	22	3.61
.2gr/100cc	1145	255	22.27
.5gr/100cc	522	271	51.92
	adenine sulfate		
.1gr/100cc	706	25	3.541
.25gr/100cc	579	26	4.49
.5gr/100cc	450	7	1.55

mum at 0.1-2.5 gr./100cc. and decline as concentration increases beyond this point. This decline may be associated with the fact that at concentration 0.5 gr./100 cultures proved to be difficult to rear, and even on 0.25 gr./100 some inhibition of the culture was noted.

The above results prompted two further experiments with *D. melanogaster* and a third involving another species, namely *D. virilis*.

Larvae of both the + Kingston and + Burlington strains were reared on dead yeast medium to which 0.15 gr. adenine/100 cc. medium and 0.2 gr. indoleacetic acid/100 cc. medium were added. Since no clear cut antagonism or other correlation in results was found when adenine and indoleacetic acid were added separately, the two were tried together. Results are presented in Table 2.

TABLE 2
Effects of 3 indoleacetic acid and adenine sulfate added to medium together on tumor incidence in + strains.

Concentration	Larvae scored	Total tumorous larvae	% tumor incidence
	+ *Kingston*		
.15gr adenine sulfate .2gr indoleacetic acid per 100cc medium	1280	27	2.109
	+ *Burlington*		
same as above	731	3	0.41

It is clear that the two chemicals combined do not show a stimulating effect of any significance in the + Burlington strain, and show only relatively little stimulating capacity in the + Kingston strain. Thus a neutralization, antagonistic balance, or other modification appears to result from combining the two in the same medium.

To ascertain whether wild strains developing tumors on indoleacetic acid or adenine would transmit a physiological or genetic "tu" modification to offspring, the following experiment was performed:

Both + Burlington and + Kingston larvae were reared on indoleacetic acid and adenine medium of concentrations which induced maximum tumor incidence. Late third instar larvae having tumors were then removed from these cultures and placed in fresh culture bottles of cornmeal-molasses agar, seeded with live yeast. Pupation occurred within a few hours, and adults emerged normally. Newly

emerged adults were transferred to new stock bottles of cornmeal medium where they were allowed to breed. As soon as the first pupae were observed in the bottles, all adults were removed. The F_1 generation was allowed to develop and complete pupation. As adults emerged they were examined for tumors. In both strains, results were negative; tumor incidence being well below 1%.

The final experiment involved the rearing of larvae of a strain of *D. virilis* on medium containing indoleacetic acid or adenine to ascertain if melanotic tumorous aggregations might be induced in other species than *D. melanogaster*.

Preliminary examination of *D. virilis* larvae reared on standard cornmeal molasses agar and on dead yeast medium revealed no tumors. Table 3 shows results when larvae were reared on dead yeast medium containing 0.25 gr. indoleacetic acid/100 cc. medium, 0.5 gr. indoleacetic acid/100 cc. medium and 0.25 gr. adenine/100 cc. medium.

TABLE 3
Effects of 3 indoleacetic acid and adenine on tumor incidence when added to culture medium of non-tumorous *D. virilis*.

Concentration	Total larvae scored	Number tumorous larvae	% tumor incidence
	3 indoleacetic acid		
.25gr/100cc	1209	11	0.91
.5gr/100cc	745	45	6.04
	adenine sulfate		
.25gr/100cc	979	9	0.92
.5gr/100cc	no growth	—	—

The results indicate that while only questionable stimulation resulted from addition of adenine, clearly demonstrable stimulation resulted from addition of indoleacetic acid, and as in *D. melanogaster* it increased as the concentration of chemical increased. Total stimulation was not as great at .5 gr./100 cc. medium, but it should be noted that this species generally does not thrive as well on the killed yeast medium as does *D. melanogaster*, and this fact may enter the picture.

Discussion

In light of certain previous reports (Hartung and Hartnett '51, Hartnett '52, Lewis '54) the results presented above suggest that the key to melanotic tumor aggregation in *Drosophila* may lie in an

analysis of amino acid and especially tryptophane utilization, or metabolism. In this analysis the role of indoleacetic acid, or the incidental appearance of indole compounds, probably in the digestive tract, should command special attention. Hartnett's report ('52) of unusually high tumor incidence obtained in a normally only moderately tumorous strain reared on a high tryptophane medium, is notable in the fact that it indicates no comparable tumor stimulation in normally non-tumorous strains used as controls. From this one might infer that the genetic "tu" factor or complex present in normally tumorous strains may express itself through a modification of normal enzyme systems having to do with the metabolism of tryptophane, either as it is digested and absorbed from food sources, or within the body of the larvae. The "modified metabolism" results in the proliferation of a substance or substances which cause the aggregation of atypical masses of blood cells. This suggestion is similar to one made by Wilson, King and Lowrie ('55). As Oftdetal ('52, '53) has pointed out, such aggregations form the cellular core of melanotic tumors.

In normally non-tumorous strains, the absence of the "tu" factor or complex presumably would allow for development of normal enzyme systems and tryptophane would be metabolized according to a normal pattern.

The present data support the view that the modification of the tryptophane metabolizing system, as proposed above, in some way may involve the production of indoleacetic acid or a closely related product. This is a phenomenon quite usual in the elaboration of plant auxins but possibly not usual in the digestion or amino acid metabolism of *Drosophila*.

That indoleacetic acid will induce tumorous aggregations in normally non-tumorous stocks, while tryptophane will not, strongly suggests the possibility that the "tu" factor may be expressed through the formation of this substance or a similar one.

The fact that no appreciable effect from feeding indoleacetic acid was noted in one of the tumorous stocks (*bw* $mt^{A}vg$) might be explained by assuming that maximal stimulation of cellular aggregation for environmental conditions of the experiment had already been achieved under genic influence. In the other stock (st sr) the increase achieved is actually quite considerable (1%-11%) even though it is below the maxima for the + Kingston and + Burlington strains. Since, as has already been pointed out, many environmental factors

are known to modify the actual incidence of tumors in normally tumorous strains, and these factors affect different strains differently, peripheral conditions of the experiment not under control may also have modified the basic response to indoleacetic acid.

Further, as Russel ('42) has shown, genetic modifiers too, play a significant role in the extent to which tumorous masses will aggregate.

One obvious weakness of the above hypothesis lies in the fact that no attempt has been made to ascertain if indoleacetic acid is actually absorbed unchanged from the digestive tract, or if incidence of tumors can in fact be correlated with amounts of indoleacetic acid in the larval body. This matter must be subjected to further investigation. The easiest approach would seem to lie, either in the direct injection of indoleacetic acid into the larval body, or in the analysis of larval homogenates for this substance.

The recent report of Harnley and co-workers ('54) is of interest in this connection. They find that tumors can be induced in normally non-tumorous strain larvae by the injection of larval extracts or tissue fluids obtained from tumor strain larvae. The report further indicates that "tu" cultures reared on cornmeal medium show a higher tumor incidence than cultures of the same strain reared on dead yeast medium, and extracts from cornmeal reared "tu" strains are far more effective in inducing tumors than are extracts made from "tu" strains reared on non-cornmeal media. In light of the present report it might be suggested that the difference in efficacy of larval homogenates from cornmeal reared and non-cornmeal reared cultures, could stem from the presence of corn, and thus large potential sources of indoleacetic acid, in the digestive tracts of the former and its absence in the latter. If such is the case, it would seem to indicate that indoleacetic acid is released from corn by the digestive process in the "tu" stocks and not by the digestive process of non-tumor stocks. No tumor induction followed injection of homogenates from normally non-tumor stocks. Since Harnley and his co-workers failed to induce tumor aggregation by injection of fluids from "tu" larvae reared on dead yeast, it is quite possible too that free indoleacetic acid does not exist in the body fluids of larvae, but that upon absorption from the digestive tract it is rapidly "bound" or metabolized. This in fact may be involved in the triggering mechanism of tumor aggregation.

Regarding the role of adenine, little can be said on the basis of present findings. Obviously, added without indoleacetic acid, adenine does not present a clear cut picture either of uniform stimulation or

of uniform inhibition. Further, the fact that quantities approximating 0.25 gr./100 cc. of medium show inhibitory effects on culture growth adds a confusing note. When adenine is added to medium with indoleacetic acid, most of the stimulating effect noted for indoleacetic acid is absent. The possible inhibitory effects of these chemicals combined in the culture medium of normally tumorous flies is presently being studied.

In conclusion it should be noted that the addition of indoleacetic acid to the culture medium of wild strains affects only the generation reared on the medium containing it. Thus the effect in a sense may be regarded as a phenocopy of the "tu" gene. The fact that 100% incidence was not achieved in the present set of experiments leaves the role of environmental or genetic "modifiers" still to be determined. However, the demonstration that tumorous aggregations can be produced in non-tumor strains by the feeding of this chemical should aid considerably in future investigation of the entire phenomenon of melanotic tumors in *Drosophila*.

Summary and Conclusions

Four strains of *Drosophila melanogaster,* two tumorous, and two non-tumorous wild, and a strain of *D. virilis* were reared on a dead yeast medium containing either 3 indoleacetic acid or adenine sulfate in varying concentrations. Addition of indoleacetic acid to the culture medium caused increased tumor incidence in one tumor strain of *D. melanogaster,* had no effect on tumor incidence in a second tumor strain of *D. melanogaster,* and caused substantial tumor incidence in the normally non-tumorous strains of both *D. melanogaster* and *D. virilis*. The stimulating effect appeared to increase as concentration of indoleacetic acid increased in the medium.

Indoleacetic acid and adenine added together to culture media failed to stimulate tumor development to any appreciable degree in non-tumor strains.

F_1 generations from wild strains of *D. melanogaster* in which tumors had been induced by indoleacetic acid, failed to show tumors when cultured on plain cornmeal-molasses medium. Thus it is concluded that the stimulating effect of indoleacetic acid in wild strains is probably in a sense a phenocopy effect of "tu" genes or complexes. No clear cut picture of stimulation or suppression resulted from the feeding of adenine though some alteration of incidence was noted.

The suggestion is made that the appearance of melanotic tumors in

Drosophila is a phenomenon centering around amino acid metabolism, particularly tryptophane, with indoleacetic acid or a related compound playing a large role in the actual abnormal aggregation of blood cells into tumor masses.

No attempt is made to analyze the role of modifying factors, environmental or genetic, in this study, but their importance is stressed in view of the failure to produce 100% incidence in the experiments reported.

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CHANGES IN THE SIZE OF CERTAIN ORGANS OF TUMOR-BEARING RATS, AND THE PROTECTIVE EFFECT OF RIBOFLAVIN IN RATS FED 4-DIMETHYLAMINO-AZOBENZENE[1]

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(Received for publication February 28, 1955)

In the course of a study on the chemical changes induced in rats bearing Walker carcinoma 256, and in rats fed p-dimethylaminoazobenzene (p-DAB), certain definite effects on the weight of some organs of precancerous and cancerous animals have been observed. In the case of the rats fed the carcinogen, we have found a marked protective effect of riboflavin when included in the diet at a high level. Riboflavin counteracts to a certain extent the effect of the p-DAB on the weight of certain organs, a phenomenon that as far as we know has not been reported before. Our studies on the effects of the Walker carcinoma 256 were made on tumors weighing up to 185 grams, and the results show a certain regularity, independent of age, sex, and strain of the host, and of the age of the tumor.

MATERIALS AND METHODS

The data on the Walker carcinoma 256 were obtained from 42 male and 38 female Wistar and Sherman rats, which were 52 to 206 days old at the time of sacrifice, bearing tumors of different ages and sizes, some of which were decidedly necrotic. The carcinoma was implanted subcutaneously into the right pectoral region. Rockland rat diet was fed ad libitum. The animals were put under slight ether anesthesia, and exsanguinated by decapitation. The tumors and organs were quickly removed, washed in cold saline, blotted and weighed.

[1] This investigation was supported by a research grant (No. PHS C-1370 (C3)) from the National Cancer Institute of the National Institutes of Health, Public Health Service, and a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

The data on the effect of p-DAB were obtained from male Sprague-Dawley rats, started on the synthetic diet when approximately 165 grams in weight, and killed at different intervals. The composition of the synthetic diet was similar to the one used by Miller and Baumann (11), but modified so as to contain 5,000 I.U. of vitamin D and 67,500 I.U. of vitamin A per kilo of diet. The protective diet contained an additional supplement of riboflavin of 66 mg/kg of diet, which provided a vitamin:carcinogen ratio proved to be highly protective by Harris and Clowes (6). The animals were divided into three groups: 24 were kept on the control diet, 34 on the low-riboflavin diet, and 8 on the high-riboflavin diet. The organs were obtained as described above.

Results and Discussion

In reporting the results we have decided to relate whenever possible the size of the organs in the experimental animals to that in controls of corresponding age and sex. We have preferred in such cases to express the experimental value as the ratio of the size of the organ calculated as per cent of the total body weight in the experimental animals to that of the controls.

1. *Experiments with p-dimethylaminoazobenzene (p-DAB)*

The feeding of p-DAB caused an increase in the size of the spleen, kidney, liver, lung, and testes. The results are summarized in Table 1, and in Fig. 1.

TABLE 1
Protective Effect of Riboflavin on the Size of Certain Organs of Rats Fed 4-Dimethylaminoazobenzene

Days on diet	No. of rats	Low-riboflavin diet Kidney	Lung	Testis	No. of rats	High-riboflavin diet Kidney	Lung	Testis
30	6	1.29[1]	1.43	1.22	2	1.25	1.27	0.99
60	4	1.34	1.42	1.25	2	1.08	1.02	1.22
100	7	1.17	1.29	1.63	2	1.08	1.13	1.77
128	9	1.24	1.58	2.30	2	1.04	1.16	2.41

[1] The size of the organs is expressed as the ratio of the per cent of body weight in the experimental animals to that in the controls.

Splenomegaly due to p-DAB has been reported by Sugiura and Rhoads (16), Hoch-Ligeti (7), Baker and Greenberg (3), Euler (5), and Cerecedo, Price, and Lombardo (4). Richardson and Borsos-Nachtnebel (13) reported splenomegaly in rats fed 3'-methyl-4-dimethylaminoazobenzene. Our observations are at variance with the

findings of Sugiura and Rhoads, of Hoch-Ligeti, and of Euler, who reported that the spleen returns to normal size upon appearance of the hepatoma. Baker and Greenberg (3) also failed to observe such an effect. As a matter of fact, we have found that there is a relationship

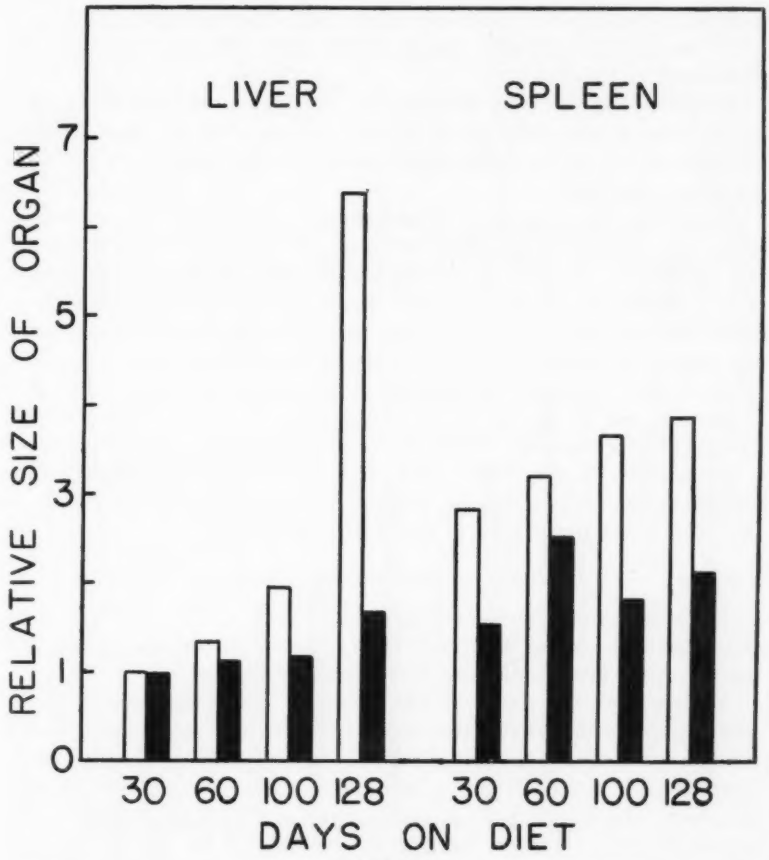

FIGURE 1

Protective effect of riboflavin on the size of the spleen and liver of rats fed 4-dimethylaminoazobenzene. The relative size is given by the ratio of the size of the organ in the experimental animals to that of corresponding controls, both expressed as per cent of body weight. Clear columns are for rats fed a low-riboflavin diet, and solid columns for those fed a high-riboflavin diet.

between the size of the spleen and that of the liver, the former reaching a value expressed as per cent of body weight which was five times as large as that of controls in a group of animals with liver weights amounting to one-third of the total body weight.

The splenomegaly manifests itself at an early stage. Four rats that had been fed the carcinogen for only 5 days had spleens 50% larger than those of the controls, and in 7 rats, after ten days, the increase amounted to 150 per cent.

In agreement with the observations of Baker and Greenberg (3), we found a hypertrophy of the testes, but contrary to them, definite increases in the organ:body weight ratios for the kidney and the lung were also observed.

Riboflavin has long been recognized as a protective factor in animals fed p-DAB, manifesting its action in a delayed appearance of the hepatoma. To us it seems significant that riboflavin also extends this protection to the size of some of the organs. Table 1 and Fig. 1 show that its inclusion in the diet at a high level lessens markedly the degree of enlargement of the liver, spleen, kidney, and lung. This effect of the vitamin is not apparent in the case of the testes. The protection can not be ascribed to a difference in the food intake, for both groups of experimental animals ate exactly the same amount of food.

In addition to the above protective effect, riboflavin lessens significantly the change in the nucleic acid:potassium ratio which is observed in the liver tissue of rats fed p-DAB (14).

2. *Experiments with Walker carcinoma 256*

The results are summarized in Table 2, and in Fig. 2. They show that the tumor causes an enlargement of the liver and the spleen, the splenomegaly being noticeable at an earlier stage.

Maurer et al. (9) report absolute weights, finding an increase in the kidney, spleen, and liver, the increase in the last two organs having been proportional to the tumor weight. Our observations confirm their findings with regard to the spleen and the liver in the cases studied by them, which included tumors that did not exceed a weight of 60 grams, but the reported increase in the size of the kidney was not observed in the present series of experiments. According to Appleman, Skavinski, and Stein (1), the UCLA fibrosarcoma and the Jensen sarcoma have no effect on the kidney.

McEwen and Haven (10) reported a maximum hepatomegaly for

TABLE 2
Effect of the Walker Carcinoma 256 on the Size of Certain Organs of the Rat

No. of rats			Organ:body ratios (per cent)			Liver[1]	
Male	Female	Tumor	Kidney	Lung	Testis	B.W.	C.W.
3	5	0.0	0.65 ± .03[2]	0.42 ± .02	0.76	1.00 ± .03	1.00 ± .03
4	3	0.4	0.64 ± .02	0.43 ± .02	0.72	1.00 ± .03	1.00 ± .03
5	0	2	0.61 ± .01	0.45 ± .03	0.89	0.95 ± .03	0.97 ± .03
3	6	4	0.66 ± .04	0.54 ± .03	1.02	1.15 ± .06	1.20 ± .07
6	4	6.5	0.67 ± .02	0.55 ± .02	0.71	1.10 ± .06	1.17 ± .06
1	3	9	0.65 ± .04	0.52 ± .07	—	1.31 ± .12	1.44 ± .13
3	3	11	0.65 ± .06	0.59 ± .09	0.96	1.22 ± .04	1.37 ± .04
4	4	14	0.62 ± .02	0.48 ± .02	0.88	1.25 ± .03	1.45 ± .04
4	4	19.5	0.60 ± .02	0.56 ± .06	0.76	1.26 ± .11	1.57 ± .14
3	3	26.5	0.50 ± .02	0.42 ± .04	0.66	1.19 ± .04	1.60 ± .10
4	1	31	0.60 ± .10	0.48 ± .10	—	1.17 ± .11	1.61 ± .14
2	2	43	0.47 ± .02	0.44 ± .05	0.57	1.18 ± .04	2.06 ± .07
Controls		—	0.69 ± .01	0.44 ± .02	0.89 ± .10	1.00 ± .02	

[1] The size of the liver is expressed as the ratio of the per cent of body weight (B.W.), or of body weight minus tumor weight (C.W.), in tumor-bearers to that in controls.
[2] Mean ± standard error.

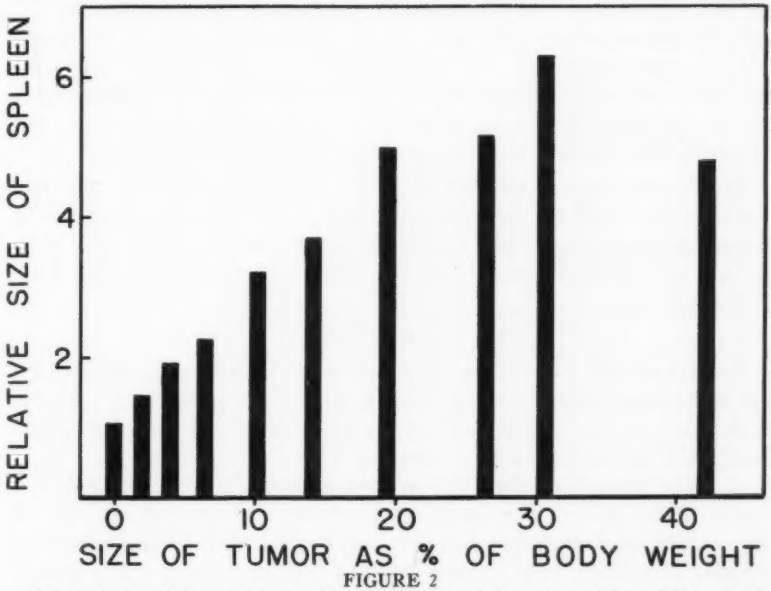

FIGURE 2

Effect of the Walker carcinoma 256 on the size of the spleen. The relative size is given by the ratio of the size of the spleen in tumor-bearers to that of corresponding controls, both expressed as per cent of total body weight. The experimental animals have been grouped as shown in Table 2.

tumors weighing around 40 grams, at which point the increase in the size of the liver, calculated as per cent of the carcass weight, amounted to about 25%. Larger tumors caused smaller increases. Calculated in this manner, our results show a decided enlargement for tumors weighing 20 grams, and a maximum enlargement for tumors of about 45 grams, with no further change up to tumors weighing 185 grams. Our observations are more in agreement with those of Yeakel (17), who studied the effects of a transplanted fibrosarcoma, and of a methylcholanthrene-induced tumor.

The splenomegaly progresses up to the point where the tumor constitutes about 15-30% of the total body weight (tumors weighing 50-80 grams), after which there occurs a decrease. Similar results have been observed by Sherman, Morton, and Mider (15). Contrary to them, we have not observed a single case of a shrunken spleen. Even in a group of four rats with tumors amounting to 43% of the body weight, the size of the spleen (expressed as per cent of the body weight) was almost five times as large as that of the controls. Splenomegaly has also been reported (8) in animals bearing the Flexner-Jobling carcinoma. In contrast, Babson (2) could not find any marked change in the spleen of rats bearing the transplanted sarcoma R-1, and Murphy and Sturm (12) reported an involution of the spleen in rats implanted with a transplantable lymphosarcoma.

We do not know of any study of the influence of the Walker carcinoma on the size of the lungs. According to our observations, the lungs undergo hypertrophy in animals with tumors constituting 4 to 20 per cent of the body weight. With larger tumors there is a return to normal size.

Summary

1. Riboflavin, at a high level, protects to a certain extent the rat against the increase in the relative size of the spleen, liver, kidney, and lung, caused by p-dimethylaminoazobenzene. In the case of the testis, this protective effect is absent.

2. The growth of the Walker carcinoma 256 causes the enlargement of the spleen, liver, and lung, but has no effect on the size of the kidney, and probably none on the testis. Marked splenohepatomegaly is present even in rats bearing tumors weighing 185 grams. In rats with tumors of a size amounting to one-fourth or more of the body weight, there is a trend for the lung to return to a normal size.

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Growth, 1955, **19**, 39-43.

BOOK REVIEW

Problems of Infancy and Childhood. (Transactions of the Seventh Conference.) Edited by Milton J. E. Senn. Josiah Macy, Jr. Foundation, New York City, 196 pages, 1953.

REVIEWED BY WILLARD C. OLSON

This seventh and final conference on the problems of infancy and childhood continues the process of interaction between theory and clinical process characteristic of the preceding ones. There is a preface by Frank Fremont-Smith. Individual reports by Sylvester, Bowlby, Caplan, and Wolf are followed by a process of group interchange. The presentation is such that the reader, himself, becomes involved in what is happening. Thus as he questions in his own mind the validity of a point that is being presented he is likely to find subsequently that the interchange has introduced a process of clarification and correction.

Sylvester starts with a report on "Developmental Truisms and Their Fate in Child Rearing-Clinical Observations". She points out the widespread seeking by parents and others for assistance on the problems of child rearing and the demand for prescriptions. Prescriptions, widely held and used, may result in mechanical answers. Thus almost any generalization becomes subject to abuse. Examples of mechanization would be conditioned response techniques; thumb guards, cuffs, etc. for thumb sucking; self-demand automats (mechanically available bottle); mechanical drill in reading; over acceptance of any formula such as protection from anxiety, permissiveness in child rearing, emphasis on precocious independence, and the early attainment of a skill because it is possible rather than needed in total development. Stress on these things may result in deprivation in other areas. Much insight is revealed in this section with its emphasis on the ever present necessity of considering individuality and variation in both the biological and social context and the interaction between the two. In child rearing education she feels that it is important to distinguish between giving general information on what children are like and telling mothers what to do.

Bowlby discusses "Some Pathological Processes Engendered by

Early Mother-Child Separation". The normal course of development involves a firm attachment to the mother in the early period of life. If separation occurs, and there is no substitute mothering, the child may never go through the "following" phase and becomes an isolate. Neurotic and psychotic character development thus may come about as a disturbance in the natural development of the relationship to the mother. Bowlby regards this inquiry into the relationship of the child to the mother as being a special case of a larger problem of ability to relate to others. There are three possible classes of deprivation: (1) a child who has no particular person with whom to make an object relation, (2) a child who moves from one relative to another at frequent intervals, and (3) a child who is taken away from the mother for a period and then returned. Bowlby's investigations have been concerned with the third type, and start with infants twelve months or more who will have developed an object relation. Important variables include heredity differences, the qualitative nature of the experience with the mother, and the way in which the removal was conducted. The circumstances during the separation experience are also important since they may vary between the presence of a substitute mother and complete absence. The transition on return again must be taken into account. Bowlby does not claim at this point that they have clear answers. They are trying rather to describe what they think happens as a preface to starting more systematic tests of the hypotheses.

Time sampling techniques with film recording provide some of the basic data. His first conclusion is that the distress upon separation is greater than most people think and has not been much studied. In a case cited as an example the evidence of separation distress is followed by withdrawal, and explosive behavior subsequently indicates the underlying tension. A frequent pattern is to scream and regress. The amount of disturbance cannot be judged alone by overt signs such as crying with someone present. There may be manifestations through immobility, lack of expression, and quiet grief when nobody is around. Thus separation can be handled by the child both by externalization and internalization. Some substitute security may be secured, for example, by cuddling a blanket or a teddy bear brought from home. During the interchange there was vigorous discussion of the question as to whether a hospital can do certain things in compensation by sensitizing staff to the human factor.

Bowlby cites Dr. Liddell's work on the response of twin goat kids, with or without their mothers, when subjected to stress. Significant differences in behavior occur in the case of twin goats under the two conditions.

To meet stress alone leads toward isolation. The generalized longitudinal sequence of events seems to be about as follows: (1) A period of protest. (2) A phase characterized, although not uniformly in all, by apathy, despair, depression, regression, absence of talking, and resumption of bed wetting. Some go into this phase and remain in it for a week or two while others scarcely seem to go into it at all. (3) The child seems to develop some type of adjustment with responses to anyone. The ordinary response of the child to his mother becomes dislocated. While some people greet this phase as evidence of happy adjustment, it may not be a long time good. The initial period of screaming subsides and in the following period there may be a repression of many memories and of the mother image.

In a separation children need their mothers and the pain of needing them and not getting them is an intense stress situation which they cannot tolerate indefinitely. In extreme cases this may go to the extent of denying the mother when seen. The breaking of this phase may be done explosively. One of the discussants points out the similarity of the more protracted hospital sequence to the one which occurs in some children when they are first left at nursery school by their mothers.

Bowlby reveals an interesting sidelight on current thought on explanatory hypotheses in a half humorous aside: "In Europe everything has been believed to be constitutional, and the influence of the environment has been discounted. In this country environment is the thing, and the constitutional aspect has been blotted out. In England we have a nice balance between the two!"

At this point in the interchange the discussants went heavily into the question of innateness citing the work of Tinbergen, Lorenz and Schneirla. The discussion next drifted to field theory and the difficulties that come about when one tries to describe a particle when it is a relationship between particles that is needed for an understanding of events. Returning to the infants the discussants next argued about the universality of generalizations when each child has had a unique history. Bowlby held out for some commonality in generalization without identity. Having struggled with emerging theory

the discussants seemed to agree that a good investigator uses his theory but never believes in it.

Caplan next reports on clinical observations on the emotional life of children in the communal settlements in Israel. The chief theme of his presentation was the difference in emphasis on communal rearing of children as opposed to emphasis on family rearing. He sees evidence of the fact that the grownup children identify with group values rather than with the values of their families under this system and in cases of conflict may support the group. Children are born in hospitals, there is no rooming in, right from birth they are separated from their mothers, when they return to the settlements the infants are housed in the communal nursery, and from then on the child is housed separately. After the first year in the communal nursery the child moves to the toddler house where he stays until the age of three and after that he moves to the kindergarten house and later to the school house. In the first year children are handled by a trained worker in groups of five to six and these groups are merged so that a worker has ten to twelve between ages one and three. Thereafter there is usually one adult for about fifteen children. The mothers have a vacation for the first six weeks after delivery and are given time off to age four or five months to feed the baby. After that feeding is taken over by the professional workers. Parents then may visit their children for two hours at the end of the day's work and for longer periods at weekends. Siblings may visit with the parents. As the child grows older, he may be taken for walks or back to the room of the parents. The task of caring for the children is regarded as one of the most important jobs in the commune. The adult founders of the "Kibbutzim" tended to belong to an extremely high intellectual class. Currently the problem of absorbing the exiles is conflicting with the ideal of "Kibbutzim". Political ideology also tends to divide.

Caplan pointed out that the above is largely the original situation which was almost universal in the settlements until some ten years ago. There is now a generation of children brought up in this manner. In the last few years, the social pattern has begun to change toward greater family identification.

Caplan observed that children below the age of from about five to seven, and particularly the toddlers, show a tremendous amount of thumb sucking, temper tantrums, lack of control over aggression,

and a high incidence of enuresis. The toddlers frequently behave like deprived children in institutions. Up to the age of six they look as though they are suffering from maternal deprivation. Above that age the incidence of signs of disturbance falls and by ten or eleven they seem to be only those typical of our culture. The young adult is remarkably non-neurotic, sociable, rather extroverted, and is prepared to sacrifice himself for his group to a notable degree. "How can one explain this extraordinary state of affairs?"

The above account appeared to serve as a real challenge to the discussants during the interchange. Discussion moved to cooperation and competition. Caplan reported that the young adults show positive evidence of cooperativeness with absence of competition among members of a group.

Caplan agreed that even under the above conditions the children form very important attachments to their parents. There is a secondary strong emotional attachment to the professional workers. There has been some experimentation recently with mixed vertical age groupings. As the children grow older the peer group becomes more and more effective as a gratifier of the needs of the individual members. The "Kibbutz" is characterized by complete co-education including the living arrangements. The members of the group tend to behave toward each other as siblings and to marry out of the group. Most of the fundamental questions raised by the Caplan report remained unresolved at the close of the discussion.

The transactions continue with "Observations of Individual Tendencies in the Second Year of Life" by Catherine M. Wolf. This is a continuation of a case study of Christina, described in earlier reports. The main lines of the development between one and two years of age are described. A report of a conference of a subcommittee on rooming-in is printed as an Appendix.

A topical and name index to the transactions from 1947-1953 enhance the usefulness of the series for the investigator or practitioner who wishes to search for particular material. A useful task for a student would be to sift the seven reports for major concepts, evidence of "creative synthesis", and the identification of hypotheses which might lend themselves to further tests.